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Proteomic analysis of *Glossina pallidipes* Salivary Gland Hypertrophy Virus virions for immune intervention in tsetse fly colonies

Henry M. Kariithi[1, 3 ‡], Ikbal Agah Ince[1, 4‡], Sjef Boeren[2], Jacques Vervoort[2],
Max Bergoin[5], Monique M. van Oers[1], Adly M. M. Abd-Alla[3], Just M. Vlak [1]*

[1]Laboratory of Virology, Wageningen University, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands, [2]Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands, [3] Insect Pest Control Laboratory, Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture, Vienna, Austria, [4]Department of Biology, Faculty of Arts and Sciences, Giresun University, 28049, Giresun, Turkey, [5] Laboratoire de Pathologie Comparée, Université Montpellier 2, Place Eugene Bataillon, 34095 Montpellier, France.

[‡]Authors contributed equally to this work
[*]Corresponding author: Tel: +31 317 483090, Fax: +31 317 484820, E-mail: just.vlak@wur.nl

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Abstract:

Many species of tsetse flies (Diptera: Glossinidae) can be infected by a virus that causes salivary gland hypertrophy (SGH). The genomes of viruses isolated from *Glossina pallidipes* (GpSGHV) and *Musca domestica* (MdSGHV) have recently been sequenced. Tsetse flies with SGH have a reduced fecundity and fertility which cause a serious problem for mass rearing in the frame of sterile insect technique (SIT) programs to control and eradicate tsetse populations in the wild. A potential intervention strategy to mitigate viral infections in fly colonies is neutralizing of the GpSGHV infection with specific antibodies against virion proteins. Two major GpSGHV virion proteins of about 130 kDa and 50 kDa, respectively, were identified by Western analysis using polyclonal rabbit antibody raised against whole GpSHGV virions. The proteome of GpSGHV, containing the antigens responsible for the immune-response, was investigated by liquid chromatography tandem mass spectrometry (LC-MS/MS) and 61 virion proteins were identified by comparison with the genome sequence. Specific antibodies were produced in rabbits against seven candidate proteins including the ORF10 / C-terminal fragment, ORF47 and ORF96 as well as proteins involved in peroral infectivity PIF-1 (ORF102), PIF-2 (ORF53), PIF-3 (ORF76) and P74 (ORF1). Antiserum against ORF10 specifically reacted to the 130 kDa protein in a Western blot analysis and to the envelope of GpSGHV using immunogold-EM. This result suggests that immune intervention of viral infections in colonies of *G. pallidipes* is a realistic option.

INTRODUCTION

Tsetse flies (Diptera: Glossinidae) are the vectors of sleeping sickness in humans (or human African trypanosomosis, HAT) and the livestock disease nagana (or African animal trypanosomosis, AAT) (Steelman, 1976; WHO, 2001). The continuing presence and advancement of tsetse flies prevents the development of sustainable and profitable livestock production systems, thus greatly influencing food production, natural-resource utilization and human settlement in almost two thirds of sub-Sahara Africa (Jordan, 1986). There are no effective vaccines against trypanosomosis and the disease is mainly managed by the prophylactic and curative treatment with trypanocidal drugs. However, there have been reports of development of resistance to the available trypanocidal drugs (Aksoy & Rio, 2005). It is generally accepted that vector control remains the most effective way of managing the disease and the sterile insect technique (SIT) that relies on the sequential release of sterile male flies in the wild has proven to be a robust technique for use in an area-wide integrated pest management (AW-IPM) approach (Hendrichs *et al.*, 2007; Vreysen *et al.*, 2000). Upon mating of the sterile males with virgin female wild flies there is no offspring which leads to a reduction in tsetse fly population density. *Glossina austeni* has been successfully eradicated from the Island of Unguja, United Republic of Tanzania using insecticide impregnated targets, insecticide “pour on” on livestock and the release of sterile insects, and efforts are being made to do the same in the Southern Rift Valley of Ethiopia (Feldmann, 2005).

Sterile males for AW-IPM programmes with an SIT component are produced in mass rearing facilities and sterilized with ionizing radiation (usually ^{60}Co or ^{137}Ce). However, the production of *Glossina pallidipes* flies is hampered by the fact that the fly colonies are contaminated by a Salivary Gland Hypertrophy Virus (GpSGHV) (Ellis and Maudlin, 1987; Jaenson, 1978 (Jura *et al.*, 1988; Jura *et al.*, 1989) which affects the productivity and fecundity of these colonies. The low productivity of these colonies makes the rearing very cumbersome and often leads to colony collapse.

GpSGHV is a rod-shaped, double-stranded, circular double-stranded DNA virus with a genome of 190 kilobase (kb) pairs and averaging 70 x 640 nm in size (Fig. 1) (Abd-Alla *et al.*, 2008; Odindo *et al.*, 1986). The presence of the virus in the salivary glands of male and female flies explains the hypertrophied appearance (hyperplasia) of these glands. The virus has also been associated with testicular degeneration and ovarian abnormalities (Kokwaro *et al.*, 1990; Sang *et al.*, 1998; Sang *et al.*, 1999). Although it is not yet clear how the fly gets infected with the virus and how exactly the virus affects the mating and feeding behavior of the fly, it has been demonstrated recently that in mass rearing facilities the virus is released from the infected fly with saliva upon blood ingestion and transmitted through the blood to other flies (Abd-Alla *et al.*, 2010). In nature, the virus is likely transmitted vertically from mother to offspring, either trans-ovum or through infected milk glands (Jura *et al.*, 1989; Sang *et al.*, 1998; Sang *et al.*, 1996) and the infection is largely asymptomatic. Similar viruses have been described from the bulb fly *Merodon equestris* (Amargier *et al.*, 1979) and the house fly *Musca domestica* (Coler *et al.*, 1993).

The entire GpSGHV genome has been sequenced (Abd-Alla *et al.*, 2008) and 160 open reading frames (ORF) have been identified. Putative functions could be assigned to only very few ORFs by blasting against databases. Most notable is the presence of homologues of the *per os* infectivity factor (PIF) genes of baculoviruses. These factors are involved in the oral transmission of baculoviruses from insect to insect. Their presence in GpSGHV suggests a similar mechanism of infection, which is in fact compatible with GpSGHV transmission via the blood meal.

One strategy to control the GpSGHV infections of *G. pallipides* flies in mass-rearing facilities is to prevent horizontal GpSGHV transmission by immune-complexing the virus in the blood meal and/or in the saliva. In this paper, we report the proteomic analysis of the GpSGHV proteome and the identification of the immune-responsive, virus-encoded proteins for development of antibodies to be used in immune intervention in order to prevent SGHV infections in *G. pallidipes* colonies.

RESULTS AND DISCUSSION

Analysis of the GpSGHV proteome

The recent sequencing of the Uganda isolate of GpSGHV (Abd-Alla *et al.*, 2008; Abd-Alla *et al.*, 2009) has provided information that greatly facilitated the assignment of ORFs for virion proteins on the genome. The major disadvantage in our study is the lack of a cell culture system for the production of GpSGHV virions which made it rather difficult to purify a large amount of GpSGHV virions with high quality. GpSGHV virions were therefore, purified from hypertrophied salivary gland cells dissected from infected tsetse flies. In addition, to minimize the disadvantage of losing the envelope protein as observed after purification of GpSGHV using sucrose gradients previously (Abd-Alla *et al.*, 2007), the virus particles were purified over a nycodenz-gradient, resulting in only a single viral band at a density of 1.153g cm^{-3} (Fig. 1A). The integrity of the virions after

Nycodenz purification was checked by the transmission electron microscopy, but they appeared to be fragile (Fig. 1B and C). It is possible that some host proteins have been co-purified, but also that they are intricately associated with virions, such as actin (Lanier & Volkman, 1998; Wang *et al.*, 2010). Separation of the purified GpSGHV proteins by gradient SDS-PAGE revealed at least 35 proteins ranging in size from 10 kDa to > 130 kDa (Fig. 2A). The most abundant proteins run at about 43 to 50 kDa (multiple bands) and 130 kDa.

The gel lane was divided into 7 slices containing proteins with a molecular mass ranging from lower than 26 kDa, 26-34 kDa, 34-43 kDa, 43-55 kDa, 55-95 kDa, 95-130 kDa to > 130 kDa, respectively (Fig. 2B) and protein extracts of each of the slices were subjected to LC/MSMS analysis. A decoy database strategy was used (Elias & Gygi, 2007) which, after applying the appropriate filters, resulted in 90 protein hits: 61 viral proteins, 28 contaminants and 1 decoy hit giving a False Discovery Rate of 1.1%. The LC-MS/MS method allowed the detection of GpSGHV proteins that were present in relatively low quantities. Fifty seven GpSGHV proteins were identified with 2 or more peptides (Table 1). Manual verification of the 4 peptides with a single identified peptide not only revealed a sufficient overlap between virtual and measured MS/MS spectra but also showed that no other peptides present in the database can explain the measured spectra.

The ORFs corresponding to 61 virion proteins were superposed on the physical map of GpSGHV (Fig. 3). The virion protein genes were almost equally distributed over both strands of the genome. Within the genome two segments encoding ORF11 to 26 and ORF114 to 139 were devoid of virion protein genes. In contrast, other segments (ORF78 to 113) were densely populated with virion protein genes (22 out of 29 = 76%). Also ORF62, a giant ORF was identified as encoding a constituent of GpSGHV virions representing a protein of about 511 kDa. Such a large virion protein is not unusual for large double stranded DNA viruses, as white spot syndrome virus has a 664 kDa virion protein which is a major nucleocapsid protein (van Hulten *et al.*, 2001; Leu *et al.*, 2005). Whereas the 511 kDa protein is probably a minor component (1.8% peptide coverage), another large protein of 127 kDa (ORF10) was found in high abundance (with 16.9% coverage) and probably represents the 130 kDa protein seen in SDS-PAGE (Fig. 2A).

Gene homology and domains analyses

Homology searches with the identified GpSGHV proteins performed against GeneBank/EMBL, Swisprot, and PIR databases, revealed that of the 61 proteins identified, 4 were unique to GpSGHV (encoded by ORFs 2, 39, 47 and 49). Neither the nucleotide sequences nor the deduced protein sequences of these genes shared significant homology to other genes/proteins identified so far. In addition, among the GpSGHV proteins identified by LC-MS/MS, five proteins had homologs in baculoviruses and nudiviruses (ORFs 1, 53, 76, 102 and 110) and one had homology to a nimavirus ORF (ORF36) (Table 1).

Among the GpSGHV virion proteins identified (Table 1) there are homologs of all four baculovirus *per os* infectivity factors (PIF-1, PIF-2, PIF-3 and PIF-0/P74). These factors, encoded by ORFs 1, 53, 76 and 102, are thought to be involved in the oral infection process of baculovirus occlusion derived virions by binding to midgut epithelial cells (Slack & Arif, 2007). PIF-1, PIF-2, and P74 have been shown to mediate specific binding of ODVs to midgut cells suggesting that they are directly involved in the virus host cell interaction at an initial step in infection (Ohkawa *et al.*, 2005; Kikhno *et al.*, 2002; Slack *et al.*, 2010). Although PIF-3 appears to be an ODV-associated protein (Li *et al.*, 2007), it does not appear to be involved in specific binding and its function is not known yet. The PIF proteins are absolutely essential for baculovirus oral infectivity (Kuzio *et al.*, 1989; Kikhno *et al.*, 2002; Pijlman *et al.*, 2003; Song *et al.*, 2008; Slack *et. al.*, 2010; Peng *et al.*, 2010). The presence of these PIF proteins in the GpSGHV proteome is a strong indication that they might have a similar function in GpSGHV infection following uptake via a blood meal.

Comparative proteomics of GpSGHV and MdSGHV

Recently a limited proteomic analysis has been carried out for an SGHV from the house fly *Musca domestica* (MdSGHV), the genome of which is also entirely sequenced (Garcia-Maruniak *et al.*, 2008). This virus is highly related to GpSGHV and is proposed to be accommodated in a newly proposed virus family *Hytrosaviridae* (Garcia-Maruniak *et al.*, 2009; Abd-Alla *et al.*, 2009). GpSGHV has 42 ORFs with homologs in MdSGHV (Garcia-Maruniak *et al.*, 2009). Comparative analysis of the GpSGHV virion proteins with all MdSGHV ORFs (Garcia-Maruniak *et al.*, 2008) showed that 33 out of the 61 identified GpSGHV virion proteins were homologous to 28 MdSGHV ORFs (Table 2). The difference in ORF numbers (32 compared to 28) follows from the notion that four MsSGHV ORFs have pairs of homologs in GpSGHV (see below). Of the 28 MdGSHV ORFs homologous to the GpSGHV proteome ORFs, only 12 were actually identified in the MdSGHV proteome (Garcia *et al.*, 2008).

The homology of the virion proteins and the presence of a number of proteins shared between GpSGHV and MdSGHV virions suggests that the two viruses have further properties in common, such as their virion structure and assembly mechanisms and give further credence to the presence of a common ancestor of these two viruses (Abd-Alla *et al.*, 2009). The notion that out of four putative SGHV *per os* infectivity factors only P74 was identified in the MdSGHV virion, but not PIF-1, 2 and 3, may suggest that some virion proteins may have been missed in the MdSGHV analysis (Garcia-Maruniak *et al.*, 2008).

The comparative proteomics of GpSGHV and MdSGHV showed four instances of gene duplications in GpSGHV (Table 2). GpSGHV ORF30 (133 aa) and ORF31 (285 aa) are both homologous to MdSGHV ORF82 (359 aa). The GpSGHV ORFs ORF32/33, ORF96/97 and ORF107/108 are homologous to MdSGHV ORFs 74, 25 and 33, respectively. Gene duplication is one important mechanism by which large DNA viruses increase their genome size and is a major mechanism for the acquisition of new gene functions (Long *et al.*, 2001). ORF107 and ORF108 were shown before to be the likely result of gene duplication (Garcia-Maruniak *et al.*, 2009). On the other hand, in 2 of the 4 cases (GpSGHV ORF30/31 and ORF32/33) the two GpSGHV ORFs in these pairs are homologous to different parts of the corresponding MdSGHV ORF and show no significant mutual homology within the pairs. Hence, these pairs are most likely the consequence of extra stop codons in GpSGHV or, alternatively, the result of sequencing artifacts. For ORF96 and ORF97 the situation is less clear as these ORFs show mutual similarities over 276 amino acids, but only for 41% and only when several gaps are introduced. In addition GpSGHV ORF110 has a homolog in ORF111 as a result of gene duplication (Garcia-Maruniak *et al.*, 2009), but in the current analysis only peptides derived from ORF110 were detected.

Identification of immunodominant proteins

To identify those GpSGHV virion proteins that give an immune response in rabbits, antiserum was raised against purified GpSGHV virions and tested against GpSGHV proteins in a Western analysis (Fig. 4). The antiserum reacted with several GpSGHV proteins, more specifically to a 130 kDa protein, to proteins in the 50 kDa range and to an array of proteins in the 30 kDa range. On the basis of the proteomic LC-MS/MS and the Western analyses (Table 1 and Fig. 4) ORF10 (approximately 127 kDa) and six ORFs with molecular sizes ranging between 43-50 kDa (ORFs 41, 47, 70, 96, 97 and 140) were tested as candidates for the observed immunodominant proteins in GpSGHV (Fig. 4). These ORFs were expressed in *E. coli* BL21 cells. Due to its large size, ORF10 was split into two fragments (N- and C- terminal fragments with calculated molecular sizes of approximately 69 kDa and 66 kDa, respectively). In addition, due to their assumed important role in oral infectivity and potential target for immune intervention, all the four PIF genes were selected as candidates for protein expression in bacteria. ORF1 (*p74*), ORF41, ORF70, ORF97 and the N-terminal fragment of ORF10 could not be successfully expressed in *E. coli*. Of the six proteins that were successfully made and that reacted with the rabbit antiserum against GpSGHV virions (PIF-1, PIF-2, PIF-3, ORF47, ORF96 and the C-terminal fragment of ORF10), two ORFs, ORF10 (C-terminal) and ORF96, were selected to generate mono-specific polyclonal antibodies based on the fact that these two ORFs were found to be the major viral proteins (Table 1). Antibodies raised against the proteins encoded by ORF10 (C-terminal) and ORF96 were tested against GpSGHV and homogenates of hypertrophied salivary glands of infected *G. pallipides* flies (Fig. 5A). As expected these antibodies reacted against the 130 kDa and 50 kDa band, respectively.

Immunolocalization of SGHV proteins

Transmission electron microscopy (TEM) using the antibody against the C-terminal fragment of ORF10 gave an indication that the protein is likely to be a component of the envelope of the virus (Fig. 5B-b). Immunolocalization studies of ORF96 using the specific rabbit antibody against this protein did not give conclusive evidence whether this protein is part of the virus envelope as there were no gold particles observed on the virions under the conditions used in the TEM studies. This could mean that either the ORF96 protein could have hidden epitopes or the antiserum was not suited for immuno EM. Furthermore it could be seen in the TEM that only a few GpSGHV virion rods remained entirely intact after Nycodenz-preparation. This confirms the fragile nature of GpSGHV virus and further work need to be done to study its stability under different conditions such as temperature, and especially the effects of virus handling on its infectivity to the tsetse fly. It is to be noted that a high density of gold particles were observed when antiserum against ORF10 was used, most likely on debris of the viral envelope. In the control experiments (preimmune serum), no gold particles could be seen for either the GpSGHV virion or the nucleocapsid. These studies may be direct evidence that the ORF10 could be involved functionally in the formation and/or the assembly of the GpSGHV envelope.

CONCLUDING REMARKS

Current proteomic analysis of GpSGHV allowed us to determine a total of 61 proteins. The identities of the proteins within the virion proteome revealed many candidates which provide a basis for further studies focussing on the virulence and pathogenesis of GpSGHV as well as on mechanisms of virus infection and transmission in tsetse flies. Comparison of the 61 identified GpSGHV ORFs with double-stranded DNA viruses of other virus families showed only a few homologies (5), more specifically with baculoviruses and nudiviruses. These involve the PIF proteins that are essential for oral infectivity of baculoviruses in insects. Whether they are also functional PIF proteins, remains to be investigated. The proteomic data also clearly indicated that GpSGHV has a total of 32 structural ORFs that encode proteins not encoded in MdSGHV. Twenty-nine GpSGHV ORFs have homologs in MdSGHV, and so far twelve of these have also been identified in the MdSGHV proteome. In addition MdSGHV has a number of virion proteins that do not have homologs in GpSGHV. Therefore, this analysis further supports the placement of these two members of the newly-proposed *Hytrosaviridae* family in two separate genera (proposed names *Glossinavirus* and *Muscavirus*) (Abd-Alla *et al.*, 2009). The role of these virion proteins in virion structure and infectivity will be the subject of future investigations.

Proteomic and immunolocalization data indicated that the ORF10 protein is abundant and present on the virion envelope. In the light of these findings, the ORF10 protein would be a good target for studies to mitigate infections of tsetse colonies by the

SGHV. The approach would be to supplement the blood meal with the ORF10 antibody or by immunizing cattle who are the blood donors in tsetse fly rearing systems.

MATERIAL AND METHODS

Preparation of virus particles and analysis by LC-MS/MS

GpSGHV virions were purified from hypertrophied salivary glands collected from a *G. pallidipes* colony maintained at the Entomology Unit of the FAO/IAEA Agriculture & Biotechnology Laboratory, Seibersdorf, Austria. Hypertrophied salivary glands were collected from dissected flies, homogenized in Tris buffer (50 mM, pH 7.8), and clarified twice by centrifugation for 10 min at 3000 g. The supernatant was layered on a 10-60% linear Nycodenz gradient and centrifuged for 1h at 27,000 g. The viral band was taken and washed in Tris buffer and centrifuged for 1h at 150,000 g. The viral pellet was resuspended in Tris buffer.

The purified virus particles were solubilized in 2x concentrated Laemmli buffer, and fractionated by SDS-PAGE (12%). Fermentas PageRuler Prestained Marker proteins were used. The gel was stained with colloidal blue and the gel lane containing the virion proteins was excised into seven contiguous sections spanning the complete gel lane based on a comparison with molecular markers.

In-gel protein digestions and peptide extractions were performed at 25°C according to a method described (Ince *et al.*, 2010). The peptides resulting from this digestion were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) by injecting 18 µl of sample on a 0.10 x 32-mm Prontosil 300-5-C18H pre-concentration column

(Bischoff, Germany) at a flow of 6 µl/min for 5 min. Peptides were eluted from the pre-concentration column onto a 0.10 x 200-mm Prontosil analytical column 300-3-C18H (Bischoff) with an acetonitrile (ACN; HPLC grade) gradient at a flow of 0.5 µl/min for 50 min. The gradient consisted of 10 to 35% (v/v) acetonitril increased in water with 1 ml/L formic acid in 50 min. As a subsequent cleaning step, the ACN concentration was increased to 80% (v/v) in 3 min (with 20% water and 1 ml/L formic acid in both the ACN and the water). Between the pre-concentration and analytical column, an electrospray potential of 3.5 kV was applied directly to the eluent via a solid 0.5 mm platina electrode fitted into a P875 Upchurch microT. Full scan positive mode Fourier transform mass spectra (FTMS) were measured between mass-to-charge ratios of 380 and 1400 with a LTQ-Orbitrap spectrometer (Thermo electron, San Jose, CA, USA).

Tandem mass spectrometry (MS/MS) scans of the four most abundant doubly and triply charged peaks in the FTMS scan were recorded in a data dependent mode in the linear trap (MS/MS threshold = 10.000). All MS/MS spectra obtained with each run were analyzed with Bioworks 3.1.1 software (Thermo Fisher Scientific, Inc.). A maximum of a single differential modification allowed per peptide was set for oxidation of methionines and de-amidation of asparagine and glutamine residues. Carboxamidomethylation of cysteines was set as a fixed modification. Trypsin specificity was set to fully enzymatic and a maximum of 3 missed cleavages with monoisotopic precursor and fragment ions. The mass tolerance for peptide precursor ions was set to 10 parts per million and for MS/MS fragment ions to 0.5 Da.

A *Glossina pallidipes* salivary gland hypertrophy virus protein database was used for the analysis (EF568108; created February 25, 2008; downloaded from www.ncbi.nlm.nih.gov/sites/entrez) after adding a list of commonly observed contaminants like: BSA (P02769, bovine serum albumin precursor), trypsin (P00760, bovine), trypsin (P00761, porcin), keratin K22E (P35908, human), keratin K1C9 (P35527, human), keratin K2C1 (P04264, human) and keratin K1CI (P35527, human). A decoy database was created by adding the reversed sequences using the program SequenceReverser from the MaxQuant package (Cox *et al.*, 2008).

To identify the proteins in the GpSGHV virions, the spectra obtained from the LC-MS/MS were searched against the GpSGHV ORF database using Bioworks 3.3.1. The peptide identifications obtained were filtered in Bioworks with the following filter criteria: $\Delta Cn > 0.08$, $Xcorr > 1.5$ for charge state 2+, $Xcorr > 3.3$ for charge state 3+ and $Xcorr > 3.5$ for charge state 4+ (Peng *et al.*, 2003). Only those proteins that showed a Bioworks Score factor (Sf) larger then 0.9 were considered.

Detection of immunodominant protein candidates

Following separation by 12% SDS-PAGE, proteins of purified GpSGHV particles were transferred to Immobilon-P membranes. Membranes were blocked overnight by incubation with 1% low fat milk and 0.05% Tween-20 in PBS (137 mM NaCL, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.76 mM KH_2PO_4 pH 7.4) at room temperature. Membranes were washed once with 0.2% low fat milk in PBS-tween-20 for 5 min, incubated with rabbit primary antibody (anti-GpSGHV; diluted 1/500, see below) at room temperature for 30 min, washed three times with PBS-Tween-20, and further incubated for 30 min in goat anti-rabbit IgG-Alkaline phosphatase conjugate (Promega) diluted 1:3000. Blots were washed three times with alkaline phosphatase buffer (0.1 M Tris-HCl, 5mm $MgCl_2$, pH 9.5) and stained with 1% of Nitro-Blue Tetrazolium Chloride/5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt (NBT/BCIP) in alkaline phosphatase buffer.

Selection of open reading frames, PCR amplification and gene cloning

Candidate open reading frames (ORFs) for protein expression were selected based on the molecular masses of LC-MS/MS identified proteins and the immunoblot analyses. Viral DNA was extracted from purified virus as previously reported (Abd-Alla *et al.*, 2007), and approximately 5 ng of the DNA was used as template. PCR amplifications were performed with HF Phusion *Taq* DNA polymerase (Finnzymes), using the reaction mixture recommended by the supplier. The primers were designed to amplify the hydrophilic regions of the selected ORFs (Supplementary Table S1) and were used at a final concentration of 0.2 mM. The PCR conditions were 98°C for 30 sec; 98ºC for 10s, 59ºC for 20s, and 72ºC for 30 s/kbp for 25 to 30 cycles; and finally 72ºC for 5 min. The PCR products were individually inserted into pJET1.2/blunt cloning vector (CloneJET^{TM} PCR Cloning kit, Fermentas). The resulting recombinant plasmids were purified with homemade GF/F columns as described in Borodina *et al.*, (2003) and the inserts were sequenced to confirm the sequences. The inserted DNA fragments were re-cloned into pET28a(+) (Sambrook *et al.*, 1989) at the multiple cloning site.

GpSGHV proteins: expression, purification and production of antisera

Escherichia coli BL21 (DE3) cells were transformed with the pET28-derived plasmids to express the cloned genes according to the pET system manual (Novagen). The bacteria expressing the viral genes were sonicated in Laemmli sample buffer (Laemmli, 1970) and purified using preparative SDS-PAGE (Model 491 Prep Cell, Bio-Rad Laboratories) according to manufacturer’s instructions. Ten μl of each recombinant protein was analyzed on 12 % SDS-PAGE followed by silver staining according to standard protocols. The purity and quantity were verified with Coomassie Blue staining and with Western blotting, using specific immune serum directed against His-tag. To reduce the amount of SDS in the samples, each protein fraction was concentrated with CENTRIPREP® YM-10 centrifugal membranes (Amicon bioseparations).

Antisera were prepared against the purified proteins (proteins encoded by ORF96 and the C-terminal fragment of ORF10) by injecting rabbits with 0.4-0.8 mg of the recombinant protein emulsified in Freund incomplete adjuvant. Two booster injections were given at 2-week intervals. Another two antisera were prepared against the P74 protein (ORF1) using the synthetic oligopeptides LYEHSKDEDGVYHRA-C (amino acids 114 to 128) and C-SEENKIASIDDKEQF (amino acids 612-626) (Pacific Immunology Corp, Ramona, CA 92065). Polyclonal antibody against the whole GpSGHV particles was collected from rabbit used for several months to feed tsetse flies in CIRAD, France.

Electron microscopy and immunolocalization of authentic viral proteins

Aliquots (5 µl) of GpSGHV virion suspension were adsorbed to carbon-coated and ionized nickel grids (400 mesh) for 5 min at room temperature and treated for negative staining with phosphotungstic acid or for immunogold labeling. For the latter the grids were then blocked with blocking buffer (5% bovine serum albumin, 5% normal serum, 0.1% cold water skin gelatin, 10 mM phosphate buffer, 150 mM NaCl, pH 7.4) for 30 min, and incubated with primary antibody or pre-immune rabbit serum (1:20 dilution in incubation buffer) for 1.5 h at room temperature. After incubation, and after several washes, the grids were incubated with goat anti-rabbit secondary antibody conjugated with gold particles (10 nm-diameter; 1:20 dilution in incubation buffer) for 45 min at room temperature. The grids were washed extensively with incubation buffer to remove excess salt, and negatively stained with 2% sodium phosphotungstate, (pH 6.5) for 5-10 sec. The specimens examined with a transmission electron microscope (Jeol, JEM-1011, 100 kV EM).

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Figure legends

Figure 1: Purification of GpSGHV virions used for mass spectrometry analysis. Virions from hypertrophied salivary glands were purified by Nycodenz gradient centrifugation (A); the arrow indicates a single distinct band that was selected after centrifugation for 1 h at 150,000 x g. Micrographs show a negatively-stained ultra-thin section (B) and a suspension of the isolated GpSGHV (C).

Figure 2: SDS-PAGE analysis of Nycodenz-purified GpSGHV stained with Coomassie blue (A) and the relative locations of the 61 SGHV-encoded virion proteins identified by LC-MS-MS analysis (B). Adjacent numbers represent the different open reading frames (ORFs), that were detected in the seven gel sections (i to vii). The numbers in the parentheses represent the number of unique peptides used to identify the designated ORFs.

Figure 3: Positioning of the 61 virion (structural) proteins encoded by GpSGHV on the genomic map of GpSGHV (Abd-Alla *et al.*, 2008). The arrows indicate the positions and the direction of transcription for the ORFs.

Figure 4: Western blot analysis of purified GpSGHV using rabbit antiserum against the whole GpSGHV virion (dilution of 1: 500). The arrows indicate two bands (approximately 130 kDa and 50 kDa) that reacted strongly with the rabbit antiserum. Budded virus (BV) and occlusion derived virus (ODV) particles from the baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) were used as negative control.

Figure 5: Detection of ORF10 and ORF96 with specific antibodies in SGHV virions. A. Western blot analysis with specific rabbit antibodies against proteins encoded by GpSGHV ORF96 and ORF10 (diluted 1: 500). Shown in the figure are Nycodenz-purified GpSGHV virions (lanes 1), a homogenate of hypertrophied (lanes 2), and non-infected (lanes 3) tsetse fly salivary gland cells. B. Electron micrographs of GpSGHV virions using pre-immune rabbit antiserum (A) and rabbit antiserum against the C-terminal fragment of the ORF10 (B). The secondary antibody was goat anti-rabbit IgG conjugated with 10 nm of gold (Aurion).

Table 1: The GpSGHV virion proteins identified by mass spectrometry in order of descending molecular mass (511 to 10 kDa)[*].

ORF	GeneBank Accession No.	Predicted mol. mass in kDa	Protein coverage in % amino acids	No. of peptides identified	pI	Characteristics of deduced proteins	Conserved domain(s)
62	YP_001687010	511.81	1.8	7	4.70		
45	YP_001686993	200.98	4.1	7	5.94		
38	YP_001686986	136.46	42.20	130	5.91		TM
10	YP_001686958	126.96	81	305	5.60	Major protein	Coiled-coil region
40	YP_001686988	104.065	3.9	3	9.16		
83	YP_001687031	81.54	13	9	5.59	***(Md013)***	
1	YP_001686949	81.35	13.8	12	4.73	p74 *(bac)* ***(Md039)***	TM
104	YP_001687052	77.84	7	6	7.30		TM, coiled-coil region
88	YP_001687036	77.71	15.3	11	7.36		
102	YP_001687050	76.07	12.4	9	5.02	PIF-1 *(bac)*	TM, SP
71	YP_001687019	71.95	2	1	6.66	***(Md090)***	
86	YP_001687034	70.13	29.4	28	7.76		
64	YP_001687012	69.98	43.7	42	8.95	ATPase/Helicase ***(Md097)***	
108	YP_001687056	63.90	30.5	15	5.34	Cell division 48-like protein ***(Md033)***	
46	YP_001686994	61.50	37.7	24	6.48	***(Md084)***	
107	YP_001687055	59.53	45.1	31	5.39	Cell division 48-like protein ***(Md33)***	
61	YP_001687009	57.40	38.3	14	8.68		
106	YP_001687054	55.05	31.3	15	5.31		Coiled-coil region
27	YP_001686975	53.03	5.5	3	6.12	Chitinase Chit1 precursor-like protein	TM
70	YP_001687018	50.89	23.9	13	9.44		
41	YP_001686989	48.74	24.9	15	6.78		
140	YP_001687088	48.41	10	5	9.26		Coiled-coil region
47	YP_001686995	47.16	61.9	98	4.47		
97	YP_001687045	44.37	62.9	83	8.61	***(Md025)***	TM
96	YP_001687044	43.50	77.2	170	6.57	Major protein ***(Md025)***	TM, SP
44	YP_001686992	42.81	20.3	8	8.84		SP
34	YP_001686982	41.15	7.7	3	9.13		
7	YP_001686955	41.02	15.3	5	9.14		
33	YP_001686981	40.98	10.3	3	9.51		
53	YP_001687001	40.21	27.8	11	5.05	PIF-2 *(bac)*	
154	YP_001687102	40.07	8	4	7.50	***(Md071)***	
2	YP_001686950	38.64	31.8	17	8.99		
93	YP_001687041	38.51	57.8	39	9.53	***(Md022)***	TM
39	YP_001686987	37.64	4.8	1	4.47		TM, SP
52	YP_001687000	36.67	6.5	7	8.63		
105	YP_001687053	34.75	7.9	2	9.82		Coiled-coil region
31	YP_001686979	33.58	19.3	5	9.62		
113	YP_001687061	33.10	34.5	11	8.76		
50	YP_001686998	32.72	29.2	45	6.33	***(Md086)***	
94	YP_001687042	32.68	57.5	34	9.28		
72	YP_001687020	31.75	27.9	7	8.32	***(Md102)***	TM
8	YP_001686956	31.58	31.4	9	9.61		
91	YP_001687039	31.47	7.1	2	7.96		TM, SP
67	YP_001687015	30.99	47.1	30	4.55		
69	YP_001687017	30.89	41.9	49	7.41		TM, SP
32	YP_001686980	30.08	13.9	3	4.90		

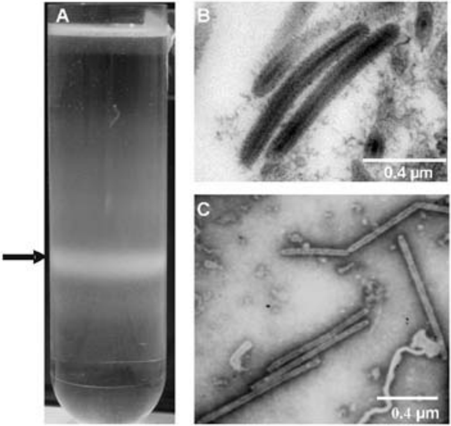
ORF	GeneBank Accession No.	Predicted mol. mass in kDa	Protein coverage in % amino acids	No. of peptides identified	pI	Characteristics of deduced proteins	Conserved domain(s)
85	YP_001687033	30.07	40	15	9.29		
78	YP_001687026	27.83	9.3	3	7.93		TM
146	YP_001687094	26.67	9	2	9.50		
84	YP_001687032	25.75	28.8	8	9.37		
76	YP_001687024	24.39	9.5	2	4.34	PIF-3 *(bac)*	TM
110	YP_001687058	23.62	27.9	4	7.71	mp-nase *(bac)*	TM
112	YP_001687060	19.05	19.2	8	4.64		TM
82	YP_001687030	18.67	23.3	3	9.84		
43	YP_001686991	16.89	69.4	28	9.91	***(Md072)***	TM
30	YP_001686978	15.91	10.5	1	10.17		
36	YP_001686984	13.79	12.2	2	5.77	Thymidylate synthase *(nima)*	
98	YP_001687046	13.52	51.3	10	10.67		
68	YP_001687016	12.64	37	6	7.63		
101	YP_001687049	12.33	62.3	37	9.56		TM, SP
49	YP_001686997	10.00	12.2	1	8.17		

[*] pI is isoelectric point, SP is signal peptide, TM is transmembrane domain; homologs with MdSGHV *(Md)*, baculovirus *(bac)* and nimavirus *(nima)* and ascoviruses are indicated.

Table 2: Proteins represented in the proteome of GpSGHV with homolog genes in MdSGHV genome. The MdSGHV homologs that were also identified in the MdSGHV proteome are in bold*.

GpSGHV ORF		**MdSGHV ORF**		**GpSGHV ORF**		**MdSGHV ORF**	
ORF	**Length**	**ORF**	**Length**	**ORF**	**Length**	**ORF**	**Length**
1 (*p74*)	696	**39 (*p74*)**	707	72	269	**102**	257
30	133	82	359	76 (*pif-3*)	211	106 (*pif-3*)	174
31	285			78	236	108	205
32	259	74	698	82	159	4	131
33	348			83	694	**13**	644
40	901	70	967	86	1779	16	1500
41	413	55	416	88	652	17	553
43	144	**72**	136	93	329	**22**	343
44	359	73	390	96	381	**25**	376
45	1728	83	1780	97	394		
46	533	**84**	509	102 (*pif-1*)	652	29 (*pif-1*)	644
50	291	**86**	381	104	660	30	692
53 (*pif-2*)	360	89 (*pif-2*)	379	107	521	**33**	497
61	494	100	455	108	545		
64	595	**97**	595	110	201	36	196
68	327	94	336	154	338	**71**	333
71	608	**90**	672				

* MdSGHV proteome data (Garcia et al., 2008; ibid, 2009). Four GpSGHV ORFs share a homolog in MdSGHV. ORF length is given in amino acids.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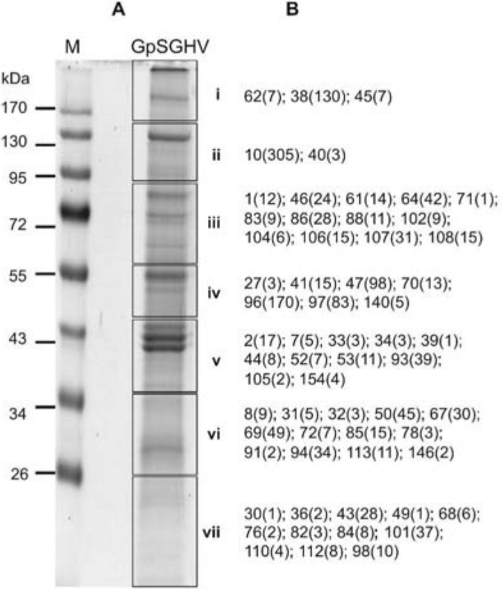
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8(9); 31(5); 32(3); 50(45); 67(30); 69(49); 72(7); 85(15); 78(3); 91(2); 94(34); 113(11); 146(2)
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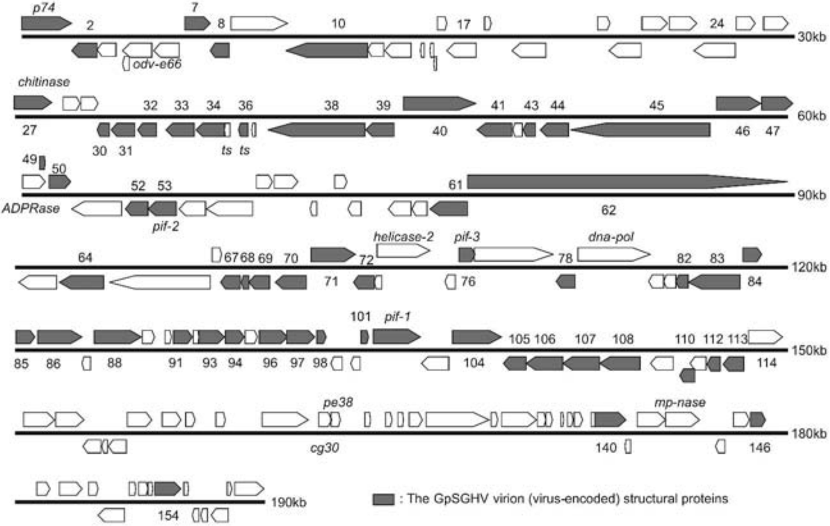
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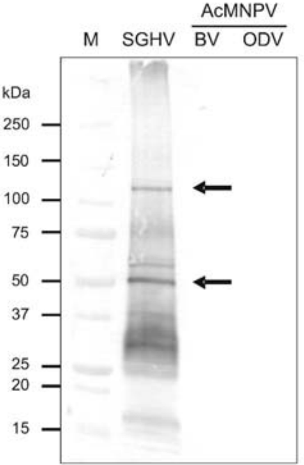
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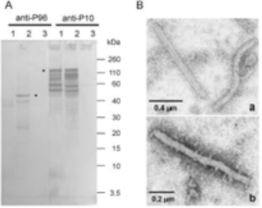
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